PhD thesis

AMPELOMYCES HYPERPARASITES IN POWDERY MILDEWS: HOST-SPECIALIZATION, INTRACELLULAR SPORULATION AND BIOCONTROL

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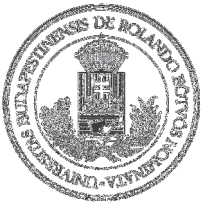

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I. Introduction

Powdery mildew fungi (the Erysiphales) are well-known pathogens of monocotyledonous and dicotyledonous plant species, including important crops. Pycnidial fungi belonging to the genus *Ampelomyces* are common intracellular mycoparasites of powdery mildews worldwide. One of the basic, and still debated, questions concerning the tritrophic relationships between host plants, powdery mildew fungi and *Ampelomyces* mycoparasites is whether *Ampelomyces* strains isolated from certain species of the Erysiphales are narrowly specialized to their original mycohosts or are generalist mycoparasites of many powdery mildew fungi. It has been shown that *Ampelomyces* populations found in apple powdery mildew (APM) (*Podosphaera leucotricha*) are genetically highly differentiated from other *Ampelomyces* populations, based on nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) sequences, sampled from several other powdery mildew species across Europe, infecting plant hosts other than apple (Szentiványi *et al*. 2005). To test the hypothesis on temporal isolation and host specialization, we investigated weather temporal isolation can play a role in the host-related restriction of gene flow.

Most studies on *Ampelomyces* dealt with strains isolated from many different powdery mildew species/genera, usually with only one or few strains from each mycohost species. Grapevine powdery mildew (*Erysiphe necator*) is one of the most important pathogens of grapevine. We chose this species to investigate the genetic diversity of *Ampelomyces* strains that naturally occur in the grapevine powdery mildew mycelium.

Ampelomyces are intracellular hyperparasites producing their pycnidia inside the powdery mildew conidiophores. The position of pycnidia in the conidiophores was suggested as a taxonomic character to distinguish groups in the genus *Ampelomyces* (Park *et al*. 2010). To investigate this presumption, we determined the localization of pycnidia of different *Ampelomyces* strains inside powdery mildew conidiophores including a special type of these structures, namely the microcyclic conidiophores.

In spite of commercial developments, biocontrol of powdery mildews on various crops was often reported as poor or inconsistent when using *Ampelomyces* as a biological control agent (BCA) in greenhouse or field trials. A possible reason of the low efficacy of the AQ10 strain and some other *Ampelomyces* strains against various powdery mildew species could be the existence of some degree of mycohost specialization presumed by some studies (e.g., Park *et al*. 2010) but rejected by others (e.g., Kiss *et al*. 2011). Grapevine powdery mildew was one of the major targets of the commercial *Ampelomyces* strain (included in the AQ10®

Biofungicide) which was applied against the conidial stage of the pathogen during the vegetation period similar to the use of chemical fungicides. Another, much less exploited strategy to control *E. necator* is to reduce the overwintering inoculum, namely the number of the sexual fruiting bodies, called chasmothecia. We carried out field experiments to investigate whether *Ampelomyces* strains applied as BCAs against chasmothecia in vineyards is an efficient way to use the hyperparasites in the control of *E. necator*.

The main objectives of the present work were as follows:

1. Are *Ampelomyces* strains isolated from different powdery mildew species strictly specialized to their original mycohosts or are generalist hyperparasites of all species of the Erysiphales?

   To answer this question we performed the following studies:

   1.1 Microsatellite markers and ITS sequences were used to investigate the genetic differentiation between *Ampelomyces* mycoparasites isolated in spring (mostly from APM) and autumn (isolates coming from many other mycohosts). Cross-inoculation experiments were performed to determine whether genetically distinct mycoparasites are narrowly specialized to their original mycohosts.

   1.2 ITS and actin gene sequences were analyzed to determine the genetic diversity of the strains isolated from *E. necator*.

2. Is the localization of intracellular pycnida of genetically different *Ampelomyces* strains influenced by the morpho-physiological patterns of powdery mildew conidiophores?

3. Can *Ampelomyces* strains parasitize powdery mildew conidiophores produced during microcyclic conidiogenesis?

4. Can *Ampelomyces* strains be used as effective biological control agents of grapevine powdery mildew?

   To answer this question we performed the following studies:

   4.1 Growth and sporulation rates of many strains were determined in culture and the mycoparasitic activities of the selected strains were determined in both the asexual and the sexual stages of *E. necator* in laboratory experiments.

   4.2 Field experiments were performed to determine the biocontrol efficacy of a selected strain against *E. necator* chasmothecia in the field.

II. Materials and methods

Ampelomyces strains

Since 2007, aerial parts of different plant species were collected in spring and autumn every year, mainly from apple tree (*Malus domestica*), wolfberry (*Lycium halimifolium*) and grapevine (*Vitis vinifera*). Pycnidia of *Ampelomyces*, produced in the powdery mildew conidiophores were collected under a stereomicroscope using sterile glass needles and then transferred to Czapek-Dox agar supplemented with 2% malt extract and 0.5% chloramphenicol.

DNA extraction

Total genomic DNA was extracted from freeze-dried mycelium of each *Ampelomyces* strain and dried apple leaf sample containing *Ampelomyces* pycnidia using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

Sequencing of the rDNA ITS region and the *act1* region of the actin gene

The nrDNA ITS region was amplified and sequenced using the fungal-specific primer pair ITS1F (Gardens and Bruns 1993) and ITS4 (White *et al*. 1990). The Act-1/ Act-5ra primer pair (Voigt and Wöstemeyer 2000) was used to amplify an approximately 850-bp-long part of the actin gene. PCRs were carried out as described in Voigt *et al*. (2005) and Park *et al*. (2010). PCR products were directly sequenced with the actin gene primers Act-1, Act-2, and Act-5ra (Voigt and Wöstemeyer 2000). Electrophoregrams were processed and analyzed with the Staden Program Package (Staden 2000).

Phylogenetic analyses

Temporal isolation: Multiple alignments of ITS sequences obtained from *Ampelomyces* strains isolated in spring and autumn were made using Multalin (Corpet 1988). For inferring phylogenies, maximum parsimony analysis was carried out using the PAUP* 4.0b10 program package (Swofford 2003) by Gábor M. Kovács .

Strains isolated from E. necator*:* The ITS sequences were aligned using MAFFT version 6 (Katoh *et al*. 2009), while the *act1* sequence alignment was completed with PRANK (Löytynoja and Goldman 2008) using the PRANKSTER interface. The alignments were checked and adjusted manually with ProSeq 2.9 (Filatov 2002). The best-fit nucleotide substitution models were selected with jModelTest 0.1.1 (Posada 2008) using Akaike information criterion (AIC). Maximum likelihood (ML) phylogenetic analyses were carried

out with the online version of PHYML 3.0 (Guindon and Gascuel 2003). In addition, Bayesian (MCMC) analysis was performed with both ITS and actin gene data sets with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using GTR nucleotide substitution model with the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu/index.aspx).

Strains tested for pycnidia-localization: Phylogenetic analyses were performed as described for the strains isolated from *E. necator*.

Microsatellite genotyping

We used five polymorphic microsatellite markers (designated AQmalus3, AQmalus4, AQmalus8, AQmalus10 and AQmalus11), developed by Harvey (2006), for genotyping *Ampelomyces* isolated from APM. PCR products were separated in 8% polyacrylamide gels and visualised by scanning the gel with a FMBIO II scanner (Hitachi Genetic Systems). The presence or absence of bands with the same size was scored by eye. A dendrogram was constructed using the unweighted pair-group method with arithmetic average (UPGMA) method with TREECON (Van de Peer and De Wachter 1994) based on the coefficient of Nei and Li.

Mycoparasitic tests

To investigate the pycnidial localization in powdery mildew conidiophores, mildew-infected tobacco, cucumber, tomato and barley plants, kept in pots in a greenhouse, as well as detached grapevine leaves, collected from potted plants kept in a greenhouse, were inoculated with the conidial suspensions of ten *Ampelomyces* strains. The concentration of the conidial suspensions was always adjusted to 10^5–10^6 conidia/ml. Inoculated plant materials, one potted plant for tobacco, cucumber, tomato and barley and 3-4 detached grapevine leaves, were kept in a climate chamber at 20°C with 16 h daily illumination period for 10 days. Following this period, detached leaves were examined for the presence of intracellular pycnidia under a stereomicroscope. Experiments were carried out three times and at least three parasitized colonies per leaf were examined using Nomarski and phase contrast optics as described by Shin and La (1993). During the microscopic survey the position of the pycnidium was determined in at least 30 conidiophores for each strain in each powdery mildew species. Data were analyzed using the UPGMA method with the SYN-TAX 2000 software package (Podani 2001) based on Jaccard's coefficient. Further statistical analyses were carried out by Imre Holb at the University of Debrecen.

Further mycoparasitic tests were performed to determine the localization of pycnidia in artificially modified conidiophores. Tobacco and grapevine powdery mildew colonies were gently brushed with a paintbrush every day to break the already developed conidiophores and prevent the maturation of the new ones. Two *Ampelomyces* strains with distinct patterns concerning the localization of their pycnidia in intact conidiophores were selected for these experiments. Microscopic surveys were done as described above.

Development of *Ampelomyces* hyphae and pycnidia was also examined in the microcyclic conidiophores of six powdery mildew species (*Golovinomyces orontii* ex tobacco *P. xanthii* ex cucumber, *Oidium neolycopersici* ex tomato, *E. necator* ex grapevine, *P. leucotricha* ex apple and *O. longipes* ex petunia) using light microscope.

Interspecific transmission of *Ampelomyces* strains under natural field conditions

We tested for transmission of *Ampelomyces* from apple powdery mildew to *G. orontii* on tobacco and *P. xanthii* on cucumber. Potted tobacco and cucumber plants artificially infected with powdery mildew were hung on two apple trees to serve as 'traps' for *Ampelomyces* mycoparasites naturally occurring in APM in spring. We exposed these same two 'trap' powdery mildew species in the same way to *Ampelomyces* parasitizing *Arthrocladiella mougeotii* on wolfberry in autumn.

To test the host-specialization of the hyperparasites in grapevine powdery mildew artificially inoculated grapevine plants with *E. necator* were placed outdoors. Trap plants were taken to the laboratory and examined for the presence of intracellular pycnidia of *Ampelomyces* in the conidiophores of *G. orontii*, *P. xanthii* and *E. necator* under a stereomicroscope. When found, *Ampelomyces* mycoparasites were isolated.

Testing *Ampelomyces* strains as potential biocontrol agents

Growth and sporulation rates in culture were determined at three temperatures (15, 20 and 25°C) in 32 strains, including the commercial strain AQ10. Based on the results, ten well-sporulating strains were selected and their **mycoparasitic activities** in grapevine powdery mildew chasmothecia and conidiophores were determined on detached leaves. As a result of these studies, a single strain, RS1-a, was selected and tested for its efficacy against grapevine powdery mildew chasmothecia in the field.

In the field experiments the following treatments were set up in autumn, after harvest, according to a complete randomized design with four replicates: i) untreated control, ii) strain RS1-a and iii) commercial product (AQ10® Biofungicide). 2 x 2 grapevine plants were

treated with a total of 2 l conidial suspensions, 10^5–10^6 conidia/ml, of strain RS1-a and AQ10, respectively. The untreated plants were sprayed with water. Two treatments were carried out in 2009 and 2010, the second treatments were done 10-14 days following the first ones in every season. Leaves were collected 10-14 days after each treatment and the presence of parasitized chasmothecia was determined under a stereomicroscope. Next spring, starting from bud break, the experimental plots in the vineyards were inspected at least once a week to determine the incidence and the severity of the symptoms of the ascosporic infections on young leaves. Data analyses were carried out at the Catholic university (Università Cattolica del Sacro Cuore) in Piacenza, Italy.

III. Results and discussion

1. Temporal isolation

Ampelomyces hyperparasites found in APM caused by *P. leucotricha* were genetically distinct from hyperparasites infecting other mycohosts responsible for powdery mildew on other plant species based on ITS sequences and microsatellite profiles. APM-*Ampelomyces* strains all carried one identical ITS haplotype, divergent from ITS haplotypes found in all but three other strains collected from powdery mildew species other than APM. Host specificity was not strict either in these *Ampelomyces* mycoparasites as revealed by our field experiments. The powdery mildew species *P. xanthii* on cucumber and *G. orontii* on tobacco became infected with both APM-*Ampelomyces* from *P. leucotricha* and *Ampelomyces* strains of two distinct ITS haplotypes often found in *A. mougeotii* infecting *L. halimifolium*.

APM overwinters in apple buds, a particular ecological situation, and it was shown that APM-*Ampelomyces* shares this overwintering site (Szentiványi and Kiss 2003). It is possible therefore that particular adaptation associated with this particular overwintering behaviour may contribute to genetic isolation between APM- and non-APM *Ampelomyces*. The life cycle of APM is mostly completed in spring and early summer while most other powdery mildew species start their life cycle later in the season, causing epidemics mostly in autumn (Szentiványi et al. 2005). Thus, the mycohost-driven genetic differentiation of that particular *Ampelomyces* lineage may be the result of differences in mycohost phenology rather than strict specialization to apple powdery mildew. We have shown that APM-*Ampelomyces* strains form a distinct clade with a single ITS haplotype and divergent microsatellite loci and alleles from the rest of the *Ampelomyces*. This is strong evidence that APM *Ampelomyces* is a cryptic species (Kiss et al. 2010).

2. Ampelomyces strains isolated from grapevine powdery mildew

The phylogenetic analyses of ITS and *act1* sequences distinguished five major clades and strains from *E. necator* that were present in all but one clade. This work showed that *Ampelomyces* strains isolated from *E. necator* are genetically diverse and there is no indication of strict mycohost associations in these strains. Trapping *Ampelomyces* mycoparasites by exposing *E. necator* on potted grapevine plants to any airborne *Ampelomyces* inoculum was successful. The four trapped strains were diverse based on both ITS and actin gene sequences. Thus, all the results showed that genetically distinct strains are able to quickly establish in grapevine powdery mildew (Pintye et al. 2012).

3. Intracellular sporulation

3.1. Pycnidial localization

The results of all the mycoparasitic tests performed with five powdery mildew species and 10 genetically different *Ampelomyces* strains showed that the morpho-physiological patterns of the powdery mildew conidiophores are those factors that strongly influence the position of pycnidia in the cells of these conidiophores. However, in certain powdery mildew species strain-specific differences were also detected in the localization of pycnidia. These *in vitro* experiments have also confirmed the lack of any strict mycohost specialization in these mycoparasites because genetically different strains infected several different powdery mildew species in addition to their original mycohosts.

3.2. Mycoparasitism of the microcyclic powdery mildew conidiophores

Microcyclic conidiogenesis was found in all the eight powdery mildew species studied (Kiss et al. 2010, Pintye et al. 2011) and intracellular hyphae as well as young pycnidia and fully mature pycnidia were found in the microcyclic conidiophores of all the powdery mildew species examined. The parasitism of the microcyclic conidiogenesis led to the rapid production of *Ampelomyces* conidia, sometimes before the start of the regular asexual reproduction in the young colony. The presence of *Ampelomyces* pycnidia in microcyclic conidiophores is a new finding and represents a previously undescribed method of accelerating asexual reproduction in this mycoparasite (Kiss et al 2010).

4. Biocontrol of grapevine powdery mildew with *Ampelomyces*

Field experiments consisted of autumn sprays in vineyards with conidial suspensions of strain RS1-a, selected during laboratory tests, and those of the AQ10 strain, with the aim to reduce

the number of chasmothecia of *Erysiphe necator*, and, thus, the amount of overwintering inocula. Spring surveys showed that the autumn treatments delayed the start of secondary infection cycles and facilitated disease control before berries gained ontogenic resistance after fruit set (Gadoury et al. 2003). The results of this work have also contributed to understanding mycohost specificity in *Ampelomyces*. No significant differences were found in the ability of a total of 32 strains, isolated from 13 powdery mildew species, including *E. necator*, to parasitize the conidial stage of *E. necator*. Moreover, RS1-a, a strain isolated from *P. pannosa* infecting rose, showed the highest rate of parasitism of *E. necator* chasmothecia. Therefore, there was no reason to select a strain isolated from *E. necator* as a potential biological control agent of grapevine powdery mildew. Our work showed that sporulation rate in culture, required for mass-production purposes, and mycoparasitic activity, but not mycohost specificity, are the key factors in selecting *Ampelomyces* strains for biocontrol of grapevine powdery mildew.

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V. List of publications

Full length papers

Pintye, A., Bereczky, Zs., Kovács, G.M., Xu, X., Legler, S.E., Váczy, Z., Váczy, K.Zs., Caffi, T., Rossi, V., Kiss, L. No indication of strict host associations in a widespread mycoparasite: Grapevine powdery mildew (*Erysiphe necator*) is attacked by phylogenetically diverse *Ampelomyces* strains in the field. *Phytopathology* 102: 707-716. **IF: 2.428**

Kiss, L., Pintye, A., Kovács, G.M., Jankovics, T., Fontaine, M., Harvey, N., Xu, X., Nicot, P.C., Bardin, M., Shykoff, J.A., Giraud, T. 2011. Temporal isolation explains host-related genetic differentiation in a group of widespread mycoparasitic fungi. *Molecular Ecology* 20: 1492–1507. **IF: 6.457**

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Short communication

Pintye, A., Legler, S.E., Kiss, L. 2011. New records of microcyclic conidiogenesis in some powdery mildew fungi. *Mycoscience* 52: 213–216. **IF: 0.774**

Conference abstracts

Legler, S.E., Caffi, T., Kiss, L., Pintye, A., Rossi., V. 2011. Methods for screening new *Ampelomyces* strains to be used as biocontrol agents against grapevine powdery mildew. *IOBC/ wprs Bulletin* 67: 149-154.

Pintye, A., Kiss, L. 2010. Microcyclic conidiogenesis, a recently discovered sporulation mechanism in powdery mildews. *International Mycological Congress 9 (IMC9) – SIG Meeting* Lecture (abstract)

Kiss, L., Pintye, A., Kovács, G.M., Fontaine, M.C., Shykoff, J.A., Giraud T., et al. 2010. Temporal isolation in the fungal world: Isolation in time explains host-related genetic differentiation in a group of widespread mycoparasitic fungi. *International Mycological Congress 9 (IMC9)* Poster (abstract)

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Legler, S.E., Caffi, T., Kiss, L., Pintye, A., Rossi., V. 2009. Methods for screening new *Ampelomyces* strains to be used as biocontrol agents against grapevine powdery mildew. *IOBC/WPRS OILB/SROP European Meeting of the Working Group Integrated Protection and Production in Viticulture*. Lecture (abstract)